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(54) Title: USES OF GREEN FLUORESCENT PROTEIN

(57) Abstract

This invention provides a cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green fluorescent protein operatively linked to a DNA sequence encoding the green fluorescent protein. This invention also provides living organisms which comprise the above-described cell. This invention also provides a method for selecting cells expressing a protein of interest which comprises: a) introducing into the cells a DNAI molecule having DNA sequence encoding the protein of interest and DNAII molecule having DNA sequence encoding a green fluorescent protein; b) culturing the introduced cells under conditions permitting expression of the green fluorescent protein and the protein of interest; and c) selecting the cultured cells which express green fluorescent protein, thereby selecting cells expressing the protein of interest. Finally, this invention provides various uses of a green fluorescent protein.

USES OF GREEN FLUORESCENT PROTEIN

This application is a continuation-in-part of United States Application Serial Nos. 08/119,678 and 08/192,274, filed September 10, 1993 and February 4, 1994, respectively, the contents of which are hereby incorporated by reference.

Background of the invention

Throughout this application various references are referred to within parenthesis. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the sequence listing and the claims.

Several methods are available to monitor gene activity and protein distribution within cells. These include the formation of fusion proteins with coding sequences for ß-galactosidase (22), and luciferases (22). The usefulness of these methods is often limited by the requirement to fix cell preparations or to add exogenous substrates or cofactors. This invention disclose a method of examining gene expression and protein localization in living cells that requires no exogenously-added compounds.

This method uses a cDNA encoding the Green fluorescent

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Protein (GFP) from the jelly fish *Aequorea victoria* (3).
In *A. victoria*, GFP absorbs energy generated by aequorin
upon the stimulation by calcium and emits a green light.

5   This   invention   discloses   that   GFP   expressed   in
prokaryotic and eukaryotic cells is capable of producing
a strong green fluorescence when excited with near UV or
blue light.   Since this fluorescence requires no
additional gene products from *A. victoria*, chromophore
10   formation is not species specific.

Summary of the invention

This invention provides a cell comprising a DNA molecule
having a regulatory element from a gene, other than a
gene encoding a green fluorescent protein operatively
linked to a DNA sequence encoding the green fluorescent
protein. This invention also provides living organisms
comprising the above-described cell.

This invention provides a method for selecting cells
expressing a protein of interest which comprises: a)
introducing into the cells a DNAI molecule having DNA
sequence encoding the protein of interest and DNAII
molecule having DNA sequence encoding a green fluorescent
protein; b) culturing the introduced cells in conditions
permitting expression of the green fluorescent protein
and the protein of interest; and c) selecting the
cultured cells which express green fluorescent protein,
thereby selecting cells expressing the protein of
interest.

This invention also provides a method for localizing a
protein of interest in a cell: a) introducing into a cell
a DNA molecule having DNA sequence encoding the protein
of interest linked to DNA sequence encoding the green
fluorescent protein such that the protein produced by the
DNA molecule will have the protein of interest fused to
the green fluorescent protein; b) culturing the cell in
conditions permitting expression of the fused protein;
c) detecting the location of the fused protein product,
thereby localizing the protein of interest.

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Brief Description of Figures

Figure 1 Expression of GFP in *E. coli*. The bacteria on the right side of the figure have the GFP expression plasmid. This photograph was taken while irradiating the agar plate with a hand-held long-wave UV source.

Figure 2 Excitation and Emission Spectra of *E. coli*-generated GFP (solid lines) and purified *A. victoria* GFP (L form; dotted lines).

Figure 3 Expression of GFP in a first stage *Caenorhabditis elegans* larva. Two touch receptor neurons (PLML and ALML) and one other neuron of unknown function (ALNL) are indicated. Processes can be seen projecting from all three cell bodies. The arrow points to the nerve ring branch from the ALML cell (out of focus). The background fluorescence is due to the animal's autofluorescence.

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Detailed Description of the Invention

Throughout this application, the following standard
abbreviations are used to indicate specific nucleotides:

C=cytosine   A=adenosine
T=thymidine   G=guanosine

This invention provides a cell comprising a DNA molecule
having a regulatory element from a gene, other than a
gene encoding a green fluorescent protein operatively
linked to a DNA sequence encoding the green fluorescent
protein.

This invention provides a cell comprising a DNA molecule
having a regulatory element from a gene, other than a
gene encoding a green fluorescent protein operatively
linked to a DNA sequence encoding the green fluorescent
protein, wherein the cell is selected from a group
consisting essentially of bacterial cell, yeast cell,
fungal cell, insect cell, nematode cell, plant or animal
cell.

Suitable animal cells include, but are not limited to
Vero cells, HeLa cells, Cos cells, CV1 cells and various
vertebral, invertebral, mammalian cells.

In an embodiment, the bacterial cell is Escherichia coli.

As used herein, "a regulatory element" from a gene is the
DNA sequence which is necessary for the transcription of
the gene.

In this invention, the term "operatively linked" means
that following such a link the regulatory element can

direct the transcription of the linked protein-coding DNA
sequence.

The gene encoding a green fluorescent protein includes
DNA molecules coding for polypeptide analogs, fragments
or derivatives of antigenic polypeptides which differ
from naturally-occurring forms in terms of the identity
or location of one or more amino acid residues (deletion
analogs containing less than all of the residues
specified for the protein, substitution analogs wherein
one or more residues specified are replaced by other
residues and addition analogs where in one or more amino
acid residues is added to a terminal or medial portion of
the polypeptides) and which share some or all properties
of naturally-occurring forms.

These DNA molecules include: the incorporation of codons
"preferred" for expression by selected mammalian or non-
mammalian hosts; the provision of sites for cleavage by
restriction endonuclease enzymes; and the provision of
additional initial, terminal or intermediate DNA
sequences that facilitate construction of expression
vectors.

As an example, plasmid pGFP10.1 codes for a mutated GFP
protein having the 80th amino acid residue as an arginine
rather than a glutamine predicted to be in native GFP
from A. victoria. This mutated protein retains the
property to fluoresce like the natural protein.

In an embodiment, the regulatory element is a promoter.
In a further embodiment, the promoter is activated by a
heavy metal. Such promoters are well-known in the art
(J.H. Freedman, L.W. Slice, A. Fire, and C.S. Rubin
(1993) Journal of Biological Chemistry, 268:2554).

In another embodiment, the promoter is that from a cytochrome P450 gene. Cytochrome P450 is well-known in the art and there are a number of P450 promoters known.

5   In still another embodiment, the promoter is that from a stress protein gene. Such stress proteins are well-known in the art (E.G. Stringham, D.K. Dixon, D. Jones and E.D. Candido (1992) *Molecular Biology of the Cell*, 3:221; and William J. Welch (May, 1993), *Scientific American*, page

10   56). In a further embodiment, the stress protein is a heat-shock protein.

This invention provides a cell comprising a DNA molecule having a regulatory element from a gene, other than a

15   gene encoding a green fluorescent protein operatively linked to a DNA sequence encoding the green fluorescent protein, wherein the promoter is from a gene necessary for the viability of a cell.

20   In another embodiment, the regulatory element is an enhancer. Enhancers are well-known in the art.

This invention provides a cell comprising a DNA molecule having a regulatory element from a gene, other than a

25   gene encoding a green fluorescent protein operatively linked to a DNA sequence encoding the green fluorescent protein, wherein the DNA sequence encodes the *Aequorea victoria* green fluorescent protein.

30   In an embodiment, the *Aequorea victoria* green fluorescent protein is cloned in a plasmid. This plasmid is a modification of the pBS(+) (formerly called Bluescribe +) vector (Stratagene®) which has inserted within it an EcoRI fragment containing the cDNA sequence of the

35   *Aequorea victoria* green fluorescent protein (as modified

herein). The fragment was obtained from λGFP10 (Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G., and Cormier, M.J., (1992) Primary structure of the *Aequorea victoria* green fluorescent protein. *Gene*, 111:229-233) by

5   amplification using the polymerase chain reaction (Saiki, R.K., Gelfand, D.H., Stoffel, S., Sharf, S.J., Higuchi, G.T., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239:487-491) with

10 primers flanking the *EcoRI* sites and subsequent digestion with *EcoRI*. The sequence of the cDNA in pGFP10.1 differs from the published sequence (5) by a change of the 80th codon of the coding sequence from CAG to CGG, a change that replaces a glutamine with arginine in the protein

15 sequence.

This pGFP10.1 plasmid was deposited on September 1, 1993 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under

20 the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganism for the Purposes of Patent Procedure. Plasmid pGFP10.1 was accorded ATCC Accession Number 75547.

25 In another embodiment, this invention provide a bacterial cell which is expressing the green fluorescent protein. In an further embodiment, the bacterial cell is an E.coli cell. In a still further embodiment, this E.coli cell is designated SMC1 (ATCC Accession No. 69554).

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This SMC1 bacterial cell was deposited on February 4, 1994, 1993 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for

35 the International Recognition of the Deposit of

Microorganism for the Purposes of Patent Procedure. Bacterial cell SMC1 was accorded ATCC Accession Number 69554.

5   This invention further provides an isolated green fluorescent protein produced from the above-described cells which comprise a DNA molecule having a regulatory element from a gene, other than a gene encoding a green fluorescent protein operatively linked to a DNA sequence

10   encoding the green fluorescent protein. This isolated green fluorescent protein can then be further modified in vitro for various uses.

       This invention disclose an efficient method for

15   expression of green fluorescent protein such that large amount of the protein could be produced. Methods to isolate expressed protein have been well-known and therefore, green fluorescent protein may be isolated easily.

20

       This invention provides a living organism comprising the cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green fluorescent protein operatively linked to a DNA sequence

25   encoding the green fluorescent protein.

       In another embodiment, the living organism is human. In another embodiment, the living organism is a mouse. The living organism may be other mammals. In addition, this

30   invention is applicable to other vertebrates, non-vertebrates and living organisms.

       In an embodiment, the living organism is C. elegans. In still another embodiment, the living organism is

35   Drosophila, zebra fish, virus or bacteriophage.

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A bacteriophage carrying the green fluorescent protein gene can infect a particular type of bacteria. The infection may be easily detected via the expression of the green fluorescent protein. Therefore, by using appropriate bacteriophages, the presence of that particular type of bacteria may be detected.

Similarly, a eucaryotic virus carrying the green fluorescent protein gene may infect a specific cell type. The infection may be easily detected by monitoring the expression of the green fluorescent protein.

Methods to introduce exogenous genetic material into a cell are well-known in the art. For example, exogenous DNA material may be introduced into the cell by calcium phosphate precipitation technology. Other technologies, such as the retroviral vector technology, electroporation, lipofection and other viral vector systems such as adeno-associated virus system, or microinjection may be used.

The above-described cells and living organisms are useful to detect effects of external stimulus to the regulatory element. The stimulus may have direct or indirect effects on the regulatory element. Such effects will be detectable through either the induction of expression and production of the green fluorescent protein or switching off the expression of the green fluorescent protein.

Cells expressing the green fluorescent proteins may be conveniently separated by a fluorescence-activated cell sorter.

These cells and organisms may be used to detect the presence of different molecules in various kinds of

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biological samples such as blood, urine or saliva. By operatively linking a regulatory element of the gene which is affected by the molecule of interest to a green fluorescent protein, the presence of the molecules will affect the regulatory element which in turn will affect the expression of the green fluorescent protein. Therefore, the above-described cells are useful for the detection of molecules. Such detection may be used for diagnostic purposes. An example of such a molecule is a hormone.

This invention provides a living organism comprising the cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green fluorescent protein operatively linked to a DNA sequence encoding the green fluorescent protein, wherein the regulatory element is for a stress protein.

This invention provides a living organism comprising the cell comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green fluorescent protein operatively linked to a DNA sequence encoding the green fluorescent protein, wherein the stress protein is a heat-shock protein.

This invention provides a method to produce green fluorescent protein comprising a) culturing the above-described cells comprising a DNA molecule having a regulatory element from a gene, other than a gene encoding a green fluorescent protein operatively linked to a DNA sequence encoding the green fluorescent protein; and b) isolating and purifying the green fluorescent protein produced from the cells. Standard methods for isolating and purifying proteins are well-known in the art. In an embodiment, the cells used for production of

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green fluorescent proteins are E. coli cells. In a
further embodiment, the E. coli cells are cultured
aerobically.

5   This invention provides a method to synthesize green
fluorescent protein comprising a) culturing the cell
designated SMC1; and b) isolating and purifying the green
fluorescent protein produced from the cell.

10   This invention provides a method for selecting cells
expressing a protein of interest which comprises: a)
introducing into the cells a DNAI molecule having DNA
sequence encoding the protein of interest and DNAII
molecule having DNA sequence encoding a green fluorescent
15   protein; b) culturing the introduced cells in conditions
permitting expression of the green fluorescent protein
and the protein of interest; and c) selecting the
cultured cells which express green fluorescent protein,
thereby selecting cells expressing the protein of
20   interest.

This invention also provides the above method, wherein
the cells are selected from a group consisting
essentially of bacterial cells, yeast cells, fungal
25   cells, insect cells, nematode cells, plant or animal
cells. Suitable animal cells include, but are not
limited to Vero cells, HeLa cells, Cos cells, CV1 cells
and various primary mammalian cells.

30   In an embodiment, DNAI and DNAII are linked. In another
embodiment, the DNA encodes the Aequorea victoria green
fluorescent protein.

This invention provides a method for localizing a protein
35   of interest in a cell which comprises: a) introducing

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into a cell a DNA molecule having DNA sequence encoding the protein of interest linked to DNA sequence encoding a green fluorescent protein such that the protein produced by the DNA molecule will have the protein of interest fused to the green fluorescent protein; b) culturing the cell in conditions permitting expression of the fused protein; and c) detecting the fused protein composed of the green fluorescent protein in the cell, thereby localizing a protein of interest in a cell.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and translation initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for translation initiation the Shine-Dalgarno sequence and the start codon ATG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon ATG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well-known in the art, for example the methods described above for constructing vectors in general.

To maximize the expression of the green fluorescent protein, the sequence flanking the translation initiation codon may be modified (reviewed by Kozak, 1984), compilation and analysis of sequences upstream from the translation start site in eukaryotic mRNAs. *Nucl. Acids. Res.* 12:857-872). A sequence may then be generated to produce higher amounts of the GFP protein.

In addition, artificial introns may be introduced so as to increase the production of the protein.

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Other special targeting sequences may be inserted into the GFP gene. One such targeting sequence is the nuclear localization signal (such as the SV40 nuclear localization signal).

The host cell of the above expression system may be selected from the group consisting of the cells where the protein of interest is normally expressed, or foreign cells such as bacterial cells (such as *E. coli*), yeast cells, fungal cells, insect cells (such as Sf9 cell in the baculovirus expression system), nematode cells, plant or animal cells, where the protein of interest is not normally expressed. Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

In an embodiment of the method for localizing a protein of interest in a cell, the DNA encoding the green fluorescent protein is from *Aequorea victoria*.

This invention provides a method for localizing a protein of interest in a cell which comprises: a) introducing into a cell a DNA molecule having DNA sequence encoding the protein of interest linked to DNA sequence encoding a green fluorescent protein such that the protein produced by the DNA molecule will have the protein of interest fused to the green fluorescent protein; b) culturing the cell in conditions permitting expression of the fused protein; and c) detecting the location of the fused protein composed of green fluorescent protein in the cell, thereby localizing a protein of interest in a cell, wherein the cell normally expressing the protein of interest.

This invention provides a method for detecting expression

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of a gene in a cell which comprises: a) introducing into
the cell a DNA molecule having DNA sequence of the gene.
linked to DNA sequence encoding a green fluorescent
protein such that the regulatory element of the gene will
5   control expression of the green fluorescent protein; b)
culturing the cell in conditions permitting expression of
the gene; and c) detecting the expression of the green
fluorescent protein in the cell, thereby indicating the
expression of the gene in the cell.

10

This invention provides a method for indicating
expression of a gene in a subject which comprises: a)
introducing into a cell of the subject a DNA molecule
having DNA sequence of the gene linked to DNA sequence
15   encoding a green fluorescent protein such that the
regulatory element of the gene will control expression of
the green fluorescent protein; b) culturing the cell in
conditions permitting expression of the fused protein;
and c) detecting the expression of the green fluorescent
20   protein in the cell, thereby indicating the expression of
the gene in the cell.

In an embodiment of the above methods, the green
fluorescent protein is the Aequorea victoria green
25   fluorescent protein.

This invention provides a method for determining the
tissue-specificity of transcription of a DNA sequence in
a subject which comprises: a) introducing into a cell of
30   the subject a DNA molecule having the DNA sequence linked
to DNA sequence encoding a green fluorescent protein such
that the DNA sequence will control expression of the
green fluorescent protein; b) culturing the subject in
conditions permitting the expression of the green
35   fluorescent protein; and c) detecting the expression of

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the green fluorescent protein in different tissue of the subject, thereby determining the tissue-specificity of the expression of the DNA sequence.

This invention provides a method for determining the presence of heavy metal in a solution which comprises: a) culturing the cell comprising a DNA molecule having a promoter from a gene, other than a green fluorescent protein operatively linked to a DNA sequence encoding the green fluorescent protein, wherein transcription at the promoter is activated by a heavy metal in the solution; and b) detecting expression of the green fluorescent protein, the expression of the green fluorescent protein indicates the presence of heavy metal.

This invention provides a method for detecting pollutants in a solution which comprises: a) culturing the cell comprising a DNA molecule having a promoter from a gene, other than a green fluorescent protein operatively linked to a DNA sequence encoding the green fluorescent protein, wherein the promoter is activated by a heavy metal or a toxic organic compound or the promoter is for a stress protein in the solution; and b) detecting expression of the green fluorescent protein, the expression of the green fluorescent protein indicates the presence of pollutants in the solution.

Finally, this invention provides a method for producing fluorescent molecular weight markers comprising: a) linking a DNA molecule encoding a green fluorescent protein with a DNA molecule encoding a known amino acid sequence in the same reading frame; b) introducing the linked DNA molecule of step a) in an expression system permitting the expression of a fluorescent protein encoded by the linked DNA molecule; and c) determining

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the molecular weight of the expressed fluorescent protein
of step b), thereby producing a fluorescent molecular
weight marker.

5   Various expression systems are known in the art. The E.
coli expression system, one of the commonly used system
is described in the following section.

The determination of molecular weight may be done by
10   comparing the expressed fluorescent protein of step b)
with known molecular weight markers. Alternatively, the
molecular weight can be predicted by calculation since
the linked DNA sequence is known (and so is the amino
acid sequence being encoded).   In an embodiment, the
15   expressed fluorescent protein is purified. The purified
fluorescent protein can be conveniently used as molecular
weight markers.

This invention will be better understood from the
20   Experimental Details which follow. However, one skilled
in the art will readily appreciate that the specific
methods and results discussed are merely illustrative of
the invention as described more fully in the claims which
follow thereafter.

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Experimental Details

A cDNA for the *Aequorea victoria* green fluorescent protein (GFP) produces a fluorescent product when expressed in prokaryotic (*Escherichia coli*) or eukaryotic (*Caenorhabditis elegans*) cells. Because exogenous substrates and cofactors are not required for this fluorescence, GFP expression can be used to monitor gene expression and protein localization in living organisms.

Light is produced by the bioluminescent jellyfish *Aequorea victoria* when calcium binds to the photoprotein aequorin (1). Although activation of aequorin in vitro or in heterologous cells produces blue light, the jellyfish produces green light. This latter light is the result of a second protein in *A. victoria* that derives its excitation energy from aequorin (2), the green fluorescent protein (GFP).

Purified GFP, a protein of 238 amino acids (3), absorbs blue light (maximally at 395 nm with a minor peak at 470 nm) and emits green light (peak emission at 509 nm with a shoulder at 540 nm) (2, 4). This fluorescence is very stable; virtually no photobleaching is observed (5). Although the intact protein is needed for fluorescence, the same absorption spectral properties found in the denatured protein are found in a hexapeptide that starts at amino acid 64 (6, 7). The GFP chromophore is derived from the primary amino acid sequence through the cyclization of Ser-dehydroTyr-Gly within this hexapeptide (7). The mechanisms that produce the dehydrotyrosine and cyclize the polypeptide to form the chromophore are unknown. To determine whether additional factors from *A. victoria* were needed for the production of the fluorescent protein, applicants tested GFP fluorescence

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in heterologous systems. Here applicants show that GFP
expressed in prokaryotic and eukaryotic cells is capable
of producing a strong green fluorescence when excited by
blue light.   Because this fluorescence requires no

5   additional gene products from A. victoria, chromophore
formation is not species specific and occurs either
through the use of ubiquitous cellular components or by
autocatalysis.

10   Expression of GFP in Escherichia coli (8) under the
control of the T7 promoter results in a readily detected
green fluorescence (9) that is not observed in control
bacteria. Upon illumination with a long-wave UV source,
fluorescent bacteria were detected on agar plates

15   containing the inducer isopropyl-β-ᴅ-thiogalactoside
(IPTG) (Fig. 1). When GFP was partially purified from
this strain (10), it was found to have fluorescence
excitation and emission spectra indistinguishable from
those of the purified native protein (Fig. 2). The

20   spectral properties of the recombinant GFP suggest that
the chromophore can form in the absence of other A.
victoria products.

Transformation of the nematode Caenorhabditis elegans

25   also resulted in the production of fluorescent GFP (11)
(Fig. 3). GFP was expressed in a small number of neurons
under the control of a promoter for the mec-7 gene. The
mec-7 gene encodes a β-tubulin (12) that is abundant in
six touch receptor neurons in C. elegans and less

30   abundant in a few other neurons (13, 14). The pattern
of expression of GFP was similar to that detected by MEC-
7 antibody or from mec-7lacZ fusions (13-15). The
strongest fluorescence was seen in the cell bodies of the
four embryonically-derived touch receptor neurons (ALML,

35   ALMR, PLML, PLMR) in younger larvae. The processes from

these cells, including their terminal branches, were
often visible in larval animals. In some newly hatched
animals, the PLM processes were short and ended in what
appeared to be prominent growth cones. In older larvae,
the cell bodies of the remaining touch cells (AVM and
PVM) were also seen; the processes of these cells were
more difficult to detect. These postembryonically-
derived cells arise during the first of the four larval
stages (16), but their outgrowth occurs in the following
larval stages (17), with the cells becoming functional
during the fourth larval stage (18). GFP's fluorescence
in these cells is consistent with these previous results:
no fluorescence was detected in these cells in newly
hatched or late first-stage larvae, but it was seen in
four of ten late second-stage larvae, all nine early
fourth-stage larvae, and seven of eight young adults
(19). In addition, moderate to weak fluorescence was
seen in a few other neurons (Fig. 3) (20). The details
of the expression pattern are being examined.

Like the native protein, GFP expressed in both *E. coli*
and *C. elegans* is quite stable (lasting at least ten
minutes) when illuminated with 450-490 nm light. Some
photobleaching occurs, however, when the cells are
illuminated with 340-390 nm or 395-440 nm light (21).

Several methods are available to monitor gene activity
and protein distribution within cells. These include the
formation of fusion proteins with coding sequences for β-
galactosidase, firefly luciferase, and bacterial
luciferase (22). Because such methods require
exogenously-added substrates or cofactors, they are of
limited use with living tissue. Because the detection of
intracellular GFP requires only irradiation by near UV or
blue light, it is not substrate limited. Thus, it should

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provide an excellent means for monitoring gene expression
and protein localization in living cells (23, 24).
Because it does not appear to interfere with cell growth
and function, GFP should also be a convenient indicator

5 of transformation and one that could allow cells to be
separated using fluorescence-activated cell sorting.
Applicants also envision that GFP can be used as a vital
marker so that cell growth (for example, the elaboration
of neuronal processes) and movement can be followed in

10 situ, especially in animals that are essentially
transparent like C. elegans and zebrafish. The
relatively small size of the protein may facilitate its
diffusion throughout the cytoplasm of extensively
branched cells like neurons and glia. Since the GFP

15 fluorescence persists after treatment with formaldehyde
(9), fixed preparations can also be examined. In
addition, absorption of appropriate laser light by GFP-
expressing cells as has been done for lucifer yellow-
containing cells (25), could result in the selective

20 killing of the cells.

Further Experiments on GFP Expression

The TU#58 plasmid, which contains the green fluorescent

25 protein (GFP) coding sequence in the pET3a expression
vector (29) was transformed into Escherichia coli strain
BLR (DE3) (A. Rocca, University of Wisconsin: cited in
the Novogen Catalogue) using procedures described
previously (29). The resulting strain (SMC3), because of

30 the reduced recombination of the host, was much more
stable for GFP expression (all the colonies on plates
with ampicillin but without the IPTG inducer (29) were
brightly fluorescent when viewed with a hand-held UV
lamp).

35

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A second construct (TU#147), similar to TU#58, was made with pET11 (A.H. Rosenberg, et al. 1987). Expression in BLR (DE3) from this plasmid was more tightly controlled; expression was seen soon after IPTG was added, but only after some time without inducer.

The SMC3 strain was used to test the requirement for aerobic growth of the bacteria for the production of a fluorescent product. Plates were grown under anaerobic conditions in a Gas-Pak container according to the instructions of the manufacturer (Becton Dickinson Microbiology Systems). Colony growth was slowed under anaerobic conditions and the resulting colones were not detectably fluorescent after at least 3 days of growth under anaerobic conditions (using the hand-held UV lab). Colonies, however, became fluorescent after a day's exposure to room air (some fluorescence was seen after a few hours).

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8. Plasmid pGFP10.1 contains the *EcoRI* fragment encoding the GFP cDNA from λgfp10 (*3*) in pBS(+) (Stratagene®). The fragment was obtained by amplification with the polymerase chain reaction [PCR; R. K. Saiki et al., *Science* **239**, 487 (1988)] with primers flanking the *EcoRI* sites and subsequent digestion with *EcoRI*. DNA was prepared by the Magic Minipreps procedure (Promega) and sequenced (after an additional ethanol precipitation) on an Applied Biosystems DNA Sequencer 370A at the DNA Sequencing facility at Columbia College of Physicians and Surgeons. The sequence of the cDNA in pGFP10.1

differs from the published sequence by a change in
codon 80 within the coding sequence from CAG to CGG,
a change that replaces a glutamine residue with
arginine [R. Heim, S. Emr, and R. Tsien (personal
communication) first alerted us to a possible
sequence change in this clone and independently
noted the same change.] This replacement has no
detectable effect on the spectral properties of the
protein (Fig. 2).

An *E. coli* expression construct was made with PCR to
generate a fragment with an NheI site at the start
of translation and an EcoRI site 5' to the
termination signal of the GFP coding sequence from
pGFP10.1.   The   5'   primer   was
ACAAAGGCTAGCAAAGGAGAAGAAC (Sequence ID No. 1) and
the 3' primer was the T3 primer (Stratagene®). The
NheI-EcoRI fragment was ligated into the similarly
cut vector pET3a [A.H. Rosenberg et al., Gene 56,
125 (1987)] by standard methods (26). The resulting
coding sequence substitutes an Ala for the initial
GFP Met, which becomes the second amino acid in the
polypeptide. The E. coli strain BL21(DE3)Lys S [F.
W. Studier and B. A. Moffat, *J. Mol. Biol.* 189, 113
(1986)] was transformed with the resulting plasmid
(TU#58) and grown at 37°C. Control bacteria were
transformed with pET3a. Bacteria were grown on
nutrient plates containing ampicillin (100 µg/ml)
and 0.8 mM IPTG. Transformed bacteria from this
transformation show green fluorescence when
irradiated with ultraviolet light. A recombinant
plasmid of this bacteria was used for the
experiments described here and the experiment in
Figure 2 and the experiment in Note 10. Several
months later, applicants noticed that the bacterial

- 25 -

colonies can be divided into two groups: i) strongly fluorescent; and 2) weakly fluorescent (applicants' believe that the weakly fluorescent may have mutated, disabled or partial or completely deleted TU#58). One strongly fluorescent colony was picked to generate the bacterial strain SMC1 (ATCC Accession No. 69554). [A similar PCR-generated fragment (see note 11) was used in applicants' *C. elegans* construct. As others are beginning to use pGFP10.1, applicants have heard that while similar PCR fragments produce a fluorescent product in other organisms (R. Heim, S. Emr, and R. Tsien, personal communication; S. Wang and T. Hazelrigg, personal communication; L. Lanini and F. McKeon, personal communication; see note 23), the *EcoRI* fragment does not (R. Heim, S. Emr, and R. Tsien, personal communication; A. Coxon, J. R. Chaillet, and T. Bestor, personal communication). These results may indicate that elements at the 5' end of the sequence or at the start of translation inhibit expression.]

9. Applicants used a variety of microscopes (Zeiss Axiophot, Nikon Microphot FXA, and Olympus BH2-RFC and BX50) equipped for epifluorescence microscopy. Usually filter sets for fluorescein isothiocyanate fluorescence were used (for example, the Zeiss filter set used a BP450-490 excitation filter, 510 nm dichroic, and either a BP515-565 or a LP520 emission filter), although for some experiments filter sets that excited at lower wavelengths were used (for example, a Zeiss filter set with BP395-440 and LP470 filters and a 460 nm dichroic or with BP340-390 and LP400 filters with a 395 nm dichroic). In some instances a xenon lamp appeared to give a more intense fluorescence than a mercury lamp when

cells were illuminated with light around 470 nm,
although usually the results were comparable. No
other attempts were made to enhance the signal (for
example, by using low intensity light cameras),
5   although this may be useful in some instances.

Previous experiments had shown that the native
protein was fluorescent after glutaraldehyde
fixation (W. W. Ward, unpub. data). S. Wang and T.
10   Hazelrigg (personal communication; 23) have found
that GFP fusion proteins in Drosophila melanogaster
are fluorescent after formaldehyde fixation.
Applicants have confirmed that fluorescence persists
after formaldehyde fixation with applicants' C.
15   elegans animals and with recombinant GFP isolated
from E. coli. The chemicals in nail polish, which
is often used to seal cover slips, however, did
appear to interfere with the C. elegans GFP
fluorescence.

20

In the applicants' initial experiments, GFP was
purified from 250 ml cultures of BL21(DE3)Lys S
bacteria containing TU#58; bacteria were grown in LB
broth (26) containing ampicillin (100 μg/ml) and 0.8
25   mM IPTG. Induction was best when IPTG was present
continually. Nevertheless, subsequent experiments
with bacterial strain SMC1 indicate that the
bacteria could not grow in the constant presence of
IPTG but can be induced by the IPTG during the log
30   phase growth. The production of fluorescent protein
is best at room temperature. Cells were washed in
4 ml of 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM
MgCl$_2$, and 10 mM dithiothreitol [A. Kumagai and W.
G. Dunphy, Cell 64, 903 (1991)] and then sonicated
35   (2 X 20 sec) in 4 ml of the same buffer containing

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0.1 mM PMSF, pepstatin A (1 μg/ml), leupeptin (1 μg/ml), and aprotinin (2 μg/ml), and centrifuged at 5,000 rpm for 5 min in the cold. The supernatant was centrifuged a second time (15,000 rpm for 15 min) and then diluted sevenfold with 10 mM Tris (pH 8.0), 10 mM EDTA, and 0.02% NaN_3. Corrected excitation and emission spectra were obtained with a SPEX FlT11 spectrofluorometer and compared with the purified L isoprotein form of GFP from *A. victoria* (M. Cutler, A. Roth, and W. W. Ward, unpub. data). The excitation spectra were measured from 300 - 500 nm with a fixed emission wavelength of 509 nm, and the emission spectra were measured from 410 - 600 nm with a fixed excitation of 395 nm. All spectra were recorded as signal-reference data (where the reference is a direct measurement of the lamp intensity with a separate photomultiplier tube) at room temperature with 1 sec integration times and 1 nm increments. The spectral band widths were adjusted to 0.94 nm for all spectra.

11. Wild-type and mutant *C. elegans* animals were grown and genetic strains were constructed according to S. Brenner, *Genetics* 77, 71 (1974).

The plasmid pGFP10.1 was used as a template for PCR (with the 5' primer GAATAAAAGCTAGCAAAGATGAGTAAAG (Sequence ID No. 2) and the 3' T3 primer) to generate a fragment with a 5' NheI site (at the start of translation) and a 3' EcoRI site (3' of the termination codon). The DNA was cut to produce an NheI - EcoRI fragment that was ligated into plasmid pPD 16.51 (12, 27), a vector containing the promoter of the *C. elegans* mec-7 gene. Wild-type *C. elegans* were transformed by coinjecting this DNA (TU#64) and

the DNA for plasmid pRF4, which contains the
dominant *rol-6(su1006)* mutation, into adult *C.*
elegans gonads as described by C. M. Mello, J. M.
Kramer, D. Stinchcomb, and V. Ambros, *EMBO J.* 10,

5   3959 (1991). A relatively stable line was isolated
(TU1710) and the DNA it carried was integrated as
described by Mitani *et al.* (15) to produce the
integrated elements *uIs3* and *uIs4* (in strains TU1754
and TU1755, respectively).

10

Living *C. elegans* animals were mounted on agar (or
agarose) pads as described (16), often with 10 mM
NaN₃ as an anesthetic (28) (another nematode
anesthetic, phenoxypropanol, quenched the

15   fluorescence) and examined with either a Zeiss
universal or axiophot microscope. For *C. elegans*,
a long-pass emission filter works best because the
animal's intestinal autofluorescence, (which
increases as the animal matures), appears yellow

20   (with band-pass filters the autofluorescence appears
green and obscures the GFP fluorescence).

Because much more intense fluorescence was seen in
uIs4 than *uIs3* animals (for example, it was often

25   difficult to see the processes of the ALM and PLM
cells in *uIs3* animals when the animals were
illuminated with a mercury lamp), the former have
been used for the observations reported here. The
general pattern of cell body fluorescence was the

30   same in both strains and in the parental,
nonintegrated strain (fluorescence in this strain
was as strong as that in the *uIs4* animals). The
uIs4 animals, however, did show an unusual
phenotype: both the ALM and PLM touch cells were

35   often displaced anteriorly. The mature cells

usually had processes in the correct positions, although occasional cells had abnormally-projecting processes. These cells could be identified as touch receptor cells, because the fluorescence was dependent on *mec-3*, a homeobox gene that specifies touch cell fate (13, 15, 18, 28). *mec-7* expression is reduced in the ALM touch cells of the head (but not as dramatically in the PLM touch cells of the tail) in *mec-3* gene mutants (13, 15). Applicants find a similar change of GFP expression in a *mec-3* mutant background for both *uIs3* and *uIs4*. Thus, GFP accurately represents the expression pattern of the *mec-7* gene. It is likely that the reduced staining in *uIs3* animals and the misplaced cells in *uIs4* animals is the result of either secondary mutations or the amount and position of the integrated DNA.

12. C. Savage, M. Hamelin, J. G. Culotti, A. Coulson, D. G. Albertson, M. Chalfie, *Genes Dev.* 3, 870 (1989).

13. M. Hamelin, I. M. Scott, J. C. Way, J. G. Culotti, *EMBO J.* 11, 2885 (1992).

14. A. Duggan and M. Chalfie, unpub. data.

15. S. Mitani, H. P. Du, D. H. Hall, M. Driscoll, M. Chalfie, *Development* 119, 773 (1993).

16. J. E. Sulston and H. R. Horvitz, *Develop. Biol.* 56, 110 (1977).

17. W. W. Walthall and M. Chalfie, *Science* 239, 643 (1988).

18. M. Chalfie and J. Sulston, *Dev. Biol.* 82, 358

- 30 -

(1981).

19. In adults, the thicker size of the animals and the more intense autofluorescence of the intestine tend to obscure these cells.

20. These include several cells in the head (including the FLP cells) and tail of newly hatched animals and the BDU cells, a pair of neurons just posterior to the pharynx. Expression of mec-7 in these cells has been seen previously (13, 15). The strongest staining of these non-touch receptor neurons are a pair of cells in the tail that have anteriorly directed processes that project along the dorsal muscle line. It is likely that these are the ALN cells, the sister cells to the PLM touch cells [J. G. White, E. Southgate, J. N. Thomson, and S. Brenner, Philos. Trans. R. Soc. Lond. B Biol. Sci. 314, 1 (1986).]

21. The photobleaching with 395-440 nm light is further accelerated, to within seconds, in the presence of 10 mM NaN$_3$, which is used as a C. elegans anesthetic (11). However, when cells in C. elegans have been photobleached, some recovery is seen within 10 min. Further investigation is needed to determine whether this recovery represents de novo synthesis of GFP. Rapid photobleaching (complete within a minute) of the green product was also seen when C. elegans was illuminated with 340-390 nm light. Unlike the photobleaching with 395-440 nm light, which abolished fluorescence produced by the 340-390 or 450-490 nm light, photobleaching with 340-390 nm light did not appear to affect the fluorescence produced by 395-490 or 450-490 nm light. Indeed,

the fluorescence produced by 450-490 nm light appeared to be more intense after brief photobleaching by 340-390 nm light. This selective photobleaching may indicate the production of more than one fluorescent product in the animal. These data on GFP fluorescence within *E. coli* and *C. elegans* is in contrast to preliminary studies that suggest that the isolated native and *E. coli* proteins are very photostable. Applicants do not know whether this in vivo sensitivity to photobleaching is a normal feature of the jellyfish protein (the fluorescence in *A. victoria* has not been examined) or results from the absence of a necessary posttranslational modification unique to *A. victoria* or nonspecific damage within the cells.

22. Reviewed in T. J. Silhavy and J. R. Beckwith, *Microbiol. Rev.* 49, 398 (1985); S. J. Gould and S. Subramani, *Anal. Biochem.* 175, 5 (1988); and G. S. A. B. Stewart and P. Williams, *J. Gen. Microbiol.* 138, 1289 (1992).

23. R. Heim, S. Emr, and R. Tsien (personal communication) have found that GFP expression in *Saccharomyces cerevisiae* can make the cells strongly fluorescent without causing toxicity. S. Wang and T. Hazelrigg (personal communication) have found that both C-terminal and N-terminal protein fusions with GFP are fluorescent in *Drosophila melanogaster*. L. Lanini and F. McKeon (personal communication) have expressed a GFP protein fusion in mammalian (COS) cells. E. Macagno (personal communication) is expressing GFP in leeches. T. Hughes (personal communication) is expressing GFP in mammalian HEK293 cells.

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24. Applicants have generated several other plasmid constructions that may be useful to investigators. These include a pBluescript II KS (+) derivative (TU#65) containing a KpnI - EcoRI fragment encoding GFP with an AgeI site 5' to the translation start and a BsmI site at the termination codon. Also available are *gfp* versions (TU#60 - TU#63) of the four *C. elegans lacZ* expression vectors (pPD16.43, pPD21.28, pPD22.04, and pPD22.11, respectively) described by Fire *et al.*, 1990 (27) except that they lack the KpnI fragment containing the SV40 nuclear localization signal.

25. J. P. Miller and A. Selverston, *Science* 206, 702 (1979).

26. J. Sambrook, E. F. Fritsch, and T. Maniatis, *Molecular cloning: A laboratory manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, (1989).

27. A. Fire, S. W. Harrison, and D. Dixon, *Gene* 93, 189 (1990).

28. J. C. Way and M. Chalfie, *Cell* 54, 5 (1988).

29. Chalfie, M., Tu. Y., Euskirchen, G., Ward, W.W., Prasher, D.C., *Science* 263, 802 (1994).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: The Trustees of Columbia University in the City of
                  New York and Woods Hole Oceanographic Institute

    (ii) TITLE OF INVENTION: USES OF GREEN FLUORESCENT PROTEIN

    (iii) NUMBER OF SEQUENCES: 2

    (iv) CORRESPONDENCE ADDRESS:
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        (C) CITY: New York
        (D) STATE: New York
        (E) COUNTRY: United States of America
        (F) ZIP: 10112

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: White, John P.
        (B) REGISTRATION NUMBER: 28,678
        (C) REFERENCE/DOCKET NUMBER: 0575/43557-B-PCT

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        (B) TELEFAX: (212) 664-0525
        (C) TELEX: 422523 COOP UI

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (vi) ORIGINAL SOURCE:
        (A) ORGANISM: Escherichia coli

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACAAAGGCTA GCAAAGGAGA AGAAC   25

- 34 -

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 28 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (ii) MOLECULE TYPE: cDNA

     (iii) HYPOTHETICAL: NO

      (vi) ORIGINAL SOURCE:
            (A) ORGANISM: Escherichia coli

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAATAAAAGC TAGCAAAGAT GAGTAAAG   28

What is claimed is:

1.   A cell comprising a DNA molecule having a regulatory
      element from a gene, other than a gene encoding a
5   green fluorescent protein operatively linked to a
      DNA sequence encoding the green fluorescent protein.

2.   A cell of claim 1, wherein the cell is selected from
      a group consisting essentially of bacterial cell,
10   yeast cell, fungal cell, insect cell, nematode cell,
      plant or animal cell.

3.   A cell of claim 1, wherein the regulatory element is
      a promoter.

15

4.   A cell of claim 3, wherein the promoter is activated
      by a heavy metal.

5.   A cell of claim 3, wherein the promoter is that from
20   a P450 gene.

6.   A cell of claim 3, wherein the promoter is from a
      gene encoding a stress protein.

25   7.   A cell of claim 6, wherein the stress protein is a
      heat-shock protein.

8.   A cell of claim 3, wherein the promoter is from a
      gene required for cell viability.

30

9.   A cell of claim 1, wherein the regulatory element is
      an enhancer.

10.   A cell of claim 1, wherein the DNA sequence encodes
35   the *Aequorea victoria* green fluorescent protein.

11.   A cell of claim 1, wherein the cell is an E. coli cell.

12.   The cell of claim 11 designated SMC1 (ATCC Accession No. 69554).

13.   An isolated green-fluorescent protein from the cell of claim 1.

14.   A living organism comprising the cell of claim 1.

15.   A living organism of claim 10, wherein the living organism is *Caenorhabditis elegans*.

16.   A living organism of claim 15, wherein the regulatory element is for a stress protein.

17.   A living organism of claim 16, wherein the stress protein is a heat-shock protein.

18.   A method to produce green fluorescent protein comprising:

    a)   culturing the cell of claim 1; and

    b)   isolating and purifying the green fluorescent protein produced from the cell.

19.   A method of claim 18, wherein the cell is an E. coli cell.

20.   A method of claim 19, wherein the cell is cultured aerobically.

21.   A method of claim 18 wherein the cell is designated

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SMC1 (ATCC Accession No. 69554).

22. A method for selecting cells expressing a protein of
     interest which comprises:

     a) introducing into the cells a DNAI molecule
         having DNA sequence encoding the protein of
         interest and DNAII molecule having DNA sequence
         encoding a green fluorescent protein;

     b) culturing the introduced cells under conditions
         permitting expression of the green fluorescent
         protein and the protein of interest; and

     c) selecting the cultured cells which express
         green fluorescent protein, thereby selecting
         cells expressing the protein of interest.

23. A method of claim 22, wherein DNAI and DNAII are
     linked.

24. A method of claim 22, wherein the cells are selected
     from a group consisting essentially of bacterial
     cells, yeast cells, fungal cells, insect cells,
     nematode cells, plant or animal cells.

25. A method of claim 22, wherein the DNAII encodes the
     Aequorea victoria green fluorescent protein.

26. A method for localizing a protein of interest in a
     cell which comprises:

     a) introducing into a cell a DNA molecule having
         DNA sequence encoding the protein of interest
         linked to DNA sequence encoding a green

fluorescent protein such that the protein produced by the DNA molecule will have the protein of interest fused to the green fluorescent protein;

b) culturing the cell under conditions permitting expression of the fused protein; and

c) detecting the location of the fused protein which composes the green fluorescent protein in the cell, thereby localizing a protein of interest in a cell.

27. A method of claim 26, wherein the cell normally expresses the protein of interest.

28. A method of claim 26, wherein the DNA encodes the Aequorea victoria green fluorescent protein.

29. A method for detecting expression of a gene in a cell which comprises:

a) introducing into the cell a DNA molecule having DNA sequence of the gene linked to DNA sequence encoding a green fluorescent protein such that the regulatory element of the gene will control expression of the green fluorescent protein;

b) culturing the cell in conditions permitting expression of the gene; and

c) detecting the expression of the green fluorescent protein in the cell, thereby indicating the expression of the gene in the cell.

30. A method for detecting expression of a gene in a subject which comprises:

    a) introducing into a cell of the subject a DNA molecule having DNA sequence of the gene linked to DNA sequence encoding a green fluorescent protein such that the regulatory element of the gene will control expression of the green fluorescent protein;

    b) culturing the cell under conditions permitting expression of the fused protein; and

    c) detecting the expression of the green fluorescent protein in the cell, thereby indicating the expression of the gene in the cell.

31. A method of claims 29 or 30, wherein the DNA encodes the *Aequorea victoria* green fluorescent protein.

32. A method for determining the tissue-specificity of the transcription of a DNA sequence in a subject which comprises:

    a) introducing into a cell of the subject a DNA molecule having the DNA sequence linked to a DNA sequence encoding a green fluorescent protein such that the DNA sequence gene will control expression of the green fluorescent protein in the subject;

    b) culturing the subject in conditions permitting the expression of the green fluorescent protein; and

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    c)   detecting the expression of the green
        fluorescent protein in different tissues of the
        subject, thereby determining the tissue-
        specificity of the DNA sequence.

33. A method for detecting heavy metal in a solution
    which comprises:

    a)   culturing the cell of claims 4 or 5 in the
        solution; and

    b)   detecting expression of the green fluorescent
        protein, the expression of the green
        fluorescent protein indicates the presence of
        heavy metal.

34. A method for detecting pollutants in a solution
    which comprises:

    a)   culturing the cell of claims 4, 5, 6 or 7 in
        the solution; and

    b)   detecting expression of the green fluorescent
        protein, the expression of the green
        fluorescent protein indicates the presence of
        a pollutant.

35. A method for producing fluorescent molecular weight
    markers comprising:

    a)   linking a DNA molecule encoding a green
        fluorescent protein with a DNA molecule
        encoding a known amino acid sequence in the
   same reading frame;

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b) introducing the linked DNA molecule of step a) in an expression system permitting the expression of a fluorescent protein encoded by the linked DNA molecule; and

5

c) determining the molecular weight of the expressed fluorescent protein of step b), thereby producing a fluorescent protein molecular weight marker.

10

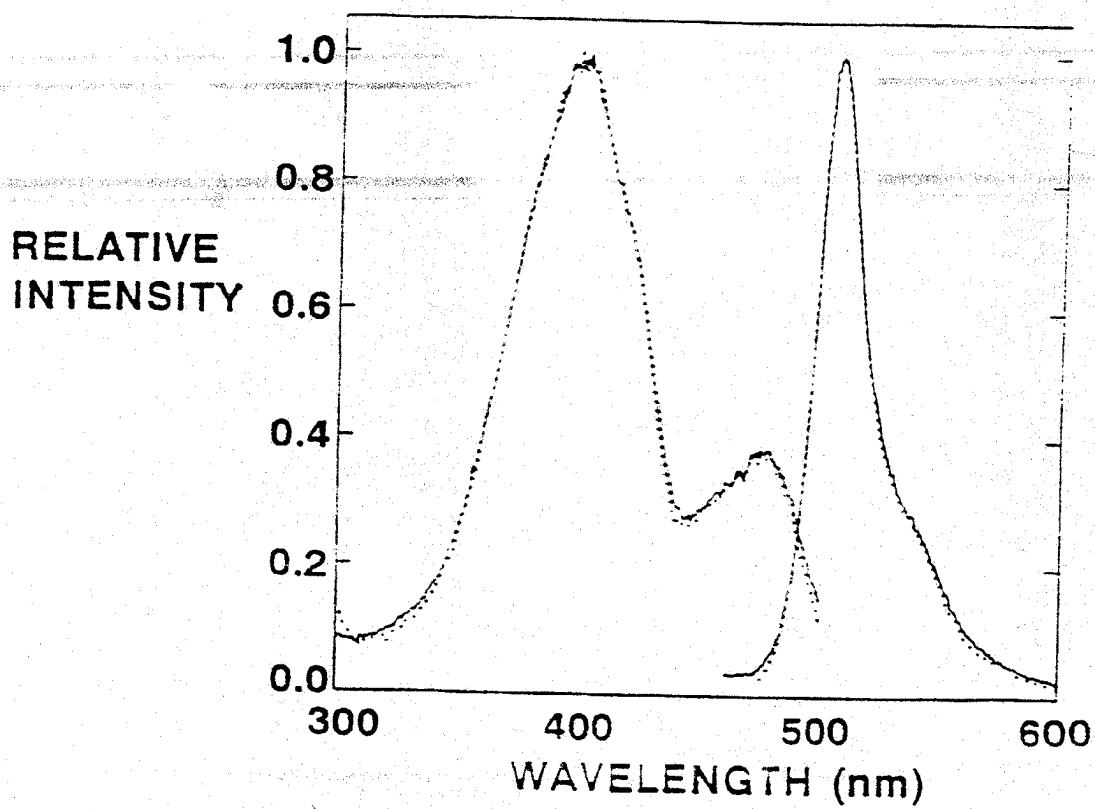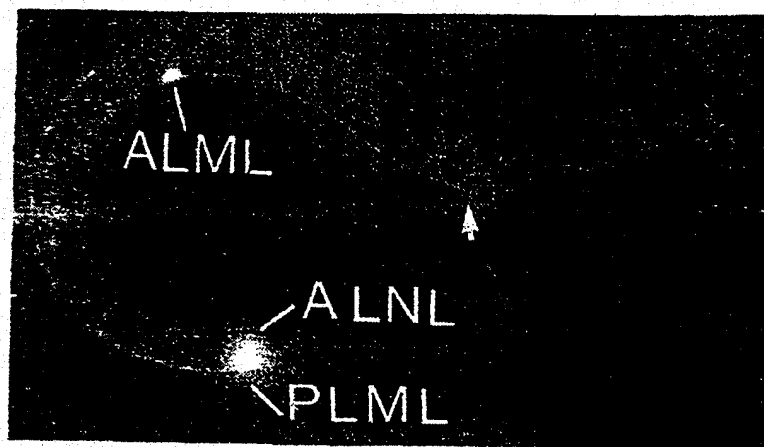36. A method of claim 35, further comprising purification of the expressed protein.
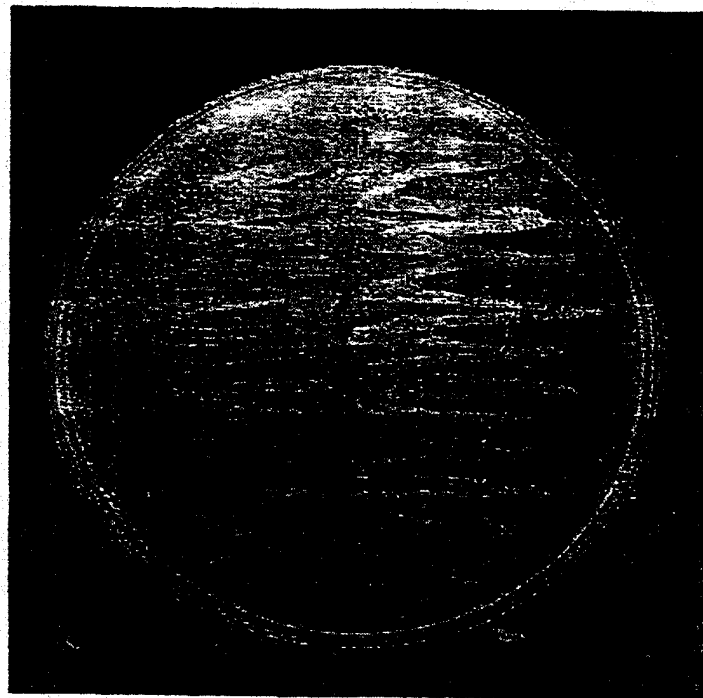
FIGURE 1

2/3

FIGURE 2

FIGURE 3

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/53; C12Q 1/00, 1/48; C12P 21/06; C12N 1/20, 5/00
US CL :435/7.6, 7.9, 15, 69.1, 69.8, 240.2, 252.3, 252.33
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.6, 7.9, 15, 69.1, 69.8, 240.2, 252.3, 252.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, STN, BIOSIS
search terms: green fluorescent protein?, aequorea, pgfp10.1, nematode?, metallothionein

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GENE, Volume 111, issued 1992, Prasher et al., "Primary structure of the *Aequorea victoria* green-fluorescent protein", pages 229-233, see especially Fig. 2.	1-36
Y	D.M. Glover, "Gene Cloning. The Mechanics of DNA Manipulation" published 1984 by Chapman and Hall (New York), pages 1-20 and 179-219, see entire document.	1-36

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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Date of the actual completion of the international search	Date of mailing of the international search report
18 DECEMBER 1994	04 JAN 1995

Name and mailing address of the ISA/US	Authorized officer
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